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Molecular epidemiology of *Mycobacterium bovis*: Usefulness in international trade

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Abstract

Tuberculosis (TB) represents a barrier for free trade of livestock between Mexico and the United States of America (US). In spite of efforts from Mexico to export TB-free animals, some of those found with TB lesions in slaughterhouses in the US are traced back to that country. Therefore, the purpose of this study was to determine, through molecular epidemiology, the most probable source of infection for cattle found with TB lesions in the US. Ninety *M. bovis* isolates, 50 from Mexico obtained from cattle in 8 different states, and 40 from the US from cattle, deer, elk and feral pigs from 7 different states were included in the study. All samples were analyzed in both laboratories, Mexico and the US, following the same protocol for molecular analysis by spoligotyping. Twenty-seven clusters, ranging from 1 to 18 genetically similar strains were found. Some clustering by country was observed, strains from cattle and deer in Michigan in the US fell into the same cluster, suggesting transmission between species. These results, combined with epidemiological information suggest that despite of the possibility that some animals with lesions in the US come from Mexico as false negatives, the US has its own source of infection, must probably in dairy cattle and wildlife. Genetic diversity of isolates from Mexico was larger than that in the US, which could be a consequence of the endemic status of the disease and the indiscriminate movement of animals between regions.

Keywords: Animal health; Bovine Tuberculosis; Molecular epidemiology; *M. bovis*; Tuberculosis

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1. Introduction

Tuberculosis is a disease with high prevalence in developing countries. According to the World Health Organization, it is estimated that there are 20 million human cases in the world, which are expected to infect 50–100 million more people annually; 80% of which are in developing countries (Murray et al., 1990). Tuberculosis (TB) in humans is primarily caused by *Mycobacterium tuberculosis*. However, other mycobacterial species, such as *Mycobacterium bovis*, the ethiological agent of bovine TB, also cause the disease (Grange and Yates, 1994). Therefore, eradicating *M. bovis* from livestock is of significant importance to human health and welfare.

Trade of livestock between Mexico and the United States (US) is important, Mexico exports to the US between 1 and 1.4 million beef calves every year. To protect its cattle industry, the US requires that all animals crossing the border are tuberculosis and brucellosis free; nevertheless, in spite of all efforts, a few animals show signs of *M. bovis* infection at slaughter. The source of infection is cause of disputes; it is possible that cattle coming from Mexico failed in reacting to the tuberculin test and are in fact false negatives, showing TB lesions at slaughter, and possibly spread the disease in cattle in the US, the reason being that some animals from the US also show TB lesions, but it is also possible that cattle in the US have their own source of infection.

PCR-based techniques have shown usefulness in relating outbreaks of TB to source of infection by strain fingerprinting (Aranaz et al., 1996; van Soolingen et al., 1994). Theories of molecular fingerprinting are that epidemiologically related isolates have similar fingerprints, that differ from those epidemiologically unrelated (Maslow and Mullingan, 1993). Therefore, a desirable characteristic for typing is related to its stability within a strain and diversity within a species. Diversity reflects the evolutionary genetic divergence arising from random non-lethal mutations that accumulate over time. However, such mutations are detectable only if they occur at sites that can be evaluated (Durr et al., 2000).

Molecular techniques based on bacterial DNA sequencing and exponential amplification of genetic targets via PCR have been shown to be useful in explaining the transmission of TB in animal populations (Collins et al., 1986, 1988; Saunders, 1991; de Lisle et al., 1990; Perumaalla et al., 1996; Szewzyk et al., 1995; van Soolingen et al., 1994; Liébana et al., 1995; Aranaz et al., 1996). Strain differentiation is the key to determine if strains of *M. bovis* from herds in close proximity are genetically similar or dissimilar, if specific geographic regions are frequent sources of infection, and if transmission occurs from one species to another.

Spoligotyping is a molecular technique that detects the presence or absence of spacers of the direct repeat (DR) locus of the *M. bovis* genome. This chromosomal region contains a large number of DRs of 36 bp interspersed with spacer DNA (DVRs) 35–41 bp in length. When the DR regions of several isolates are compared, it is observed that the order of the spacers is about the same in all isolates, but deletions and insertions of DVRs occur. The polymorphism in various isolates comprises the absence or presence of one or more DVRs. Therefore spoligotyping detects the presence or absence of spacers of known sequence, a characteristic that is used to determine genetic similarity among strains (Kamerbeek et al., 1997).

Although better methods to differentiate epidemiologically close-related strains have been described recently (Frothingham and Meeker-O'connell, 1998; Roring et al., 2004; Allix et al., 2006), spoligotyping (Kamerbeek et al., 1997) has shown to be useful in identifying sources of infection (Cousins et al., 1998), transmission of TB between species (Aranaz et al., 1996), and in identifying stability of TB strains for long periods of time in closed populations indicating that *Mycobacterium* is clonal (Perumaalla et al., 1999). In an ecological setting, spoligotyping is a rapid and inexpensive option to search for a relationship between strains.

There are reports indicating that tuberculosis in cattle from Mexico and the US have different source of infection (Perumaalla et al., 1996). Researchers speculate that transmission from non-cattle sources may be responsible for the infection of cattle in Texas. However, this report was based in a low number of isolates from each country, and included only strains from Texas. Therefore, the objective of this study was to use molecular genotyping methods to determine the most probable source of infection for cattle of both Mexican and US origin.

2. Materials and methods

2.1. Bacterial isolates

Ninety *M. bovis* isolates were included in the study: 50 from Mexico and 40 from the US. Isolates from the US included outbreaks in cattle herds from 2000 through 2005 as well as historical isolates obtained from wildlife from 1992 through 1996. Isolates from Mexico were obtained from sample-banks selected to represent isolates from different geographic locations obtained from 2003 to 2005. However, they were not a representative sample of isolates from the region involved. Mexican isolates were from cattle, mainly dairy: 11 from Nuevo Leon (NL), 9 from Tamaulipas (Tamps), 9 from Chihuahua (Chi), 8 from Sonora (Son), 5 from Baja California (BC), 4 from Jalisco (Jal), 3 from Aguascalientes (Ags), and 1 from Coahuila (Coa). The ones from the US were from 7 different states: 14 from Michigan (MI) (8 cattle, 5 deer and 1 coyote), 9 from California (CA) and 9 from Texas (TX); 3 from New Mexico (NM), 3 from Hawaii (HI) (all feral pigs), 1 from Arizona (AZ) and 1 from Montana (MT) (elk). DNA from all 90 isolates was obtained by standard procedures and split into two sets. The sets were exchanged between countries, one set was sent to the laboratory of biotechnology at INIFAP in Mexico, and the other to NVSL-APHIS-USDA in the US: both laboratories ran the same typing protocols.

2.2. Molecular analysis protocol

Spoligotyping was performed following the protocol described by Kamerbeek et al. (1997), briefly, DNA was amplified with AmpliTaq DNA Polymerase (PerkinElmer) in a 50-μl PCR mix containing 5 μl of 10× reaction buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 0.2 mM of each dNTP, 1.25 mM $MgCl_2$, 20 pmol of each primer (DRa 5′ -GGT TTT GGG TCT GAC GAC- 3′ marked with biotin at the 5′ end and DRb 5′ -CCG AGA GGG GAC GGA AAC -3) and 5 μl of template DNA. The mixture was heated in a Gene Amp PCR system 2400 (PerkinElmer) at 96 C for 3 min and subjected to 30 cycles of denaturation at 96 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 40 s, followed by a final extension at 72 C for 10 min.

The amplified DNA was visualized after electrophoresis in a SDS-polyacrylamide 12% gel stained with silver nitrate at 120 V for 90 min. Twenty microliters of PCR product was added to 150 μl of 2× SSPE-0.1% SDS and heat denatured at 99 C for 10 min, and applied to a Nylon membrane to which 37 spacer sequences from *M. tuberculosis* H37Rv and 6 spacer sequences from *M. bovis* BCG were covalently bound (ISOGEN, Maarssen, The Netherlands).

This membrane was then placed in a miniblotter MN45 (Immunetics; Cambridge, MA) in such a way that the slots were perpendicular to the line pattern of the previously applied oligonucleotides. The membranes were then incubated in 10 ml of 2× SSPE-0.5% SDS plus 5 μl of streptavidin-peroxidase conjugate for 45 min at 42 C. For detection of hybridizing DNA,

chemiluminescent ECL detection liquid (Amersham Biosciences; Piscataway, NJ) was used, followed by exposure to X-ray film (Kodak) for 12 min.

2.3. Statistical analysis

2.3.1. F-Statistics

The Fisher statistic, F_{ST}, can be interpreted as the proportion of total variance attributed to genetic differentiation between the subpopulations under comparison. A total of 136 Pairwise F_{ST} estimates were made between 16 sample sets based on state of origin; 7 from the United States (AZ, CA, HI, MI, MT, NM and TX), and 9 from Mexico (AGS, BC, BCN, CHI, COA, JAL, NL, SON and TAM). Significance was calculated by permutation of haplotypes between the sites under consideration using the Arlequin software package (Excoffier, 2000). A Bonferroni adjusted significance threshold for multiple pairwise F_{ST} comparisons was set at $\leq$0.000367 (0.05/136). Three two-way group F_{ST} comparisons were conducted using the Arlequin software package (Excoffier, 2000), and consisted of United States vs. Mexican, southwest United States (AZ, CA, and NM) samples vs. Mexican, and the remaining United States sites (HI, MI, MT, and TX) vs. Mexico. Significance was calculated by permutation, and significance level set at $\leq$0.05.

2.3.2. Phylogenetic analysis

Spoligotypes were converted to discrete character data, 0 (absent) and 1 (present), for all 43 probe hybridization positions. Relationship among *M. bovis* spoligotypes were estimated by parsimony analysis using methods described by Farris (1977) and enacted by the DOLLOP program of the PHYLIP software package (Felsenstein, 1989). Sampling error of the final parsimony-base tree was estimated by generation of 1000 pseudo replicate spoligotype datasets, wherein discrete 0 and 1 data were randomly re-sampled for each of the 43 probe hybridization positions by bootstrapping (re-sampling with replacement back into the pool of all possible data points at the relevant position). A majority-rule and strict consensus tree was assembled from all 1000 possible parsimony trees assembled by DOLLOP using the CONSENSE program by PHYLIP, and tree rooted by strain H37Rv. Bootstrap support at each Parsimony tree node is represented as the percentage of the 1000 trees in which the group consisting of the species to the right occurred.

3. Results

Eighty-four *M. bovis* strains from Mexico and the US were spoligotyped under the same protocol in two laboratories to determine genetic relationship; no DNA could be obtained from 6 of the isolates. When comparing spoligotypes from the two laboratories, 50 (59%) out of the 84 strains were identical, the rest had small differences in one or more spacer regions. From those showing differences, 17 (48%) varied at a single spacer, 10 (28%) had differences in 2 spacers, 3 (8%) varied at 3 spacer regions, and 4 (14%) were different at >3 spacers. Differences between laboratories were not random; variations in four spacers were responsible for 80% of all discordant results.

Significantly different pairwise F_{ST} estimates were observed among the United States (9 of 21 comparisons), among the Mexican samples (0 of 36 comparisons), and between United States and Mexican samples (13 of 79, $P \leq 0.000367$). Secondary two-way group F_{ST} comparisons were performed, and groups defined based upon the pairwise F_{ST} results. The first comparison was conducted between United States and Mexican samples. Additional group F_{ST} analysis was

Table 1
Analysis of molecular variance for spoligotyping patterns derived from *M. bovis* isolates originating from the United States and Mexico

Comparison[a]	F_{ST}[b]	*P*-Value
U.S. vs. MX	0.34439	<0.00001
(AZ + CA + NM) vs. MX	0.206	0.01075
(HI + MI + MT + TX) vs. MX	0.37026	<0.00001

[a] Spoligotype patterns were grouped into geographic clusters based on country and/or state of origin prior to analysis. U.S. vs. MX: = all spoligotype patterns from the United States as compared to all spoligotype patterns from Mexico. AZ + CA + NM vs. MX = spoligotype patterns from the U.S. states of Arizona, California and New Mexico compared to all isolates from Mexico. HI + MI + MT + TX vs. MX = isolates from the U.S. states of Hawaii, Michigan, Montana, and Texas compared to strains from Mexico.
[b] Fixation index representing the percent variance between the selected subpopulation and the total population.

performed for three states in the southwestern United States (AZ, CA, and NM) and Mexican samples, and between the remaining regions of the United States (HI, MI, MT, and TX) and Mexican samples (Table 1). Although still significant, less variance was observed due to genetic variability in the AZ-CA-NM isolates ($F_{ST} = 0.206$, $P = 0.01075$) versus the alternate HI-MI-MT-TX subgroup ($F_{ST} = 0.27026$, $P < 0.00001$) when compared to MX.

Phylogenetic relationships of the 84 *M. bovis* spoligotype patterns from Mexico and the US are shown in Fig. 1. Spoligotypes obtained were typical of *M. bovis*, with absence of spacers

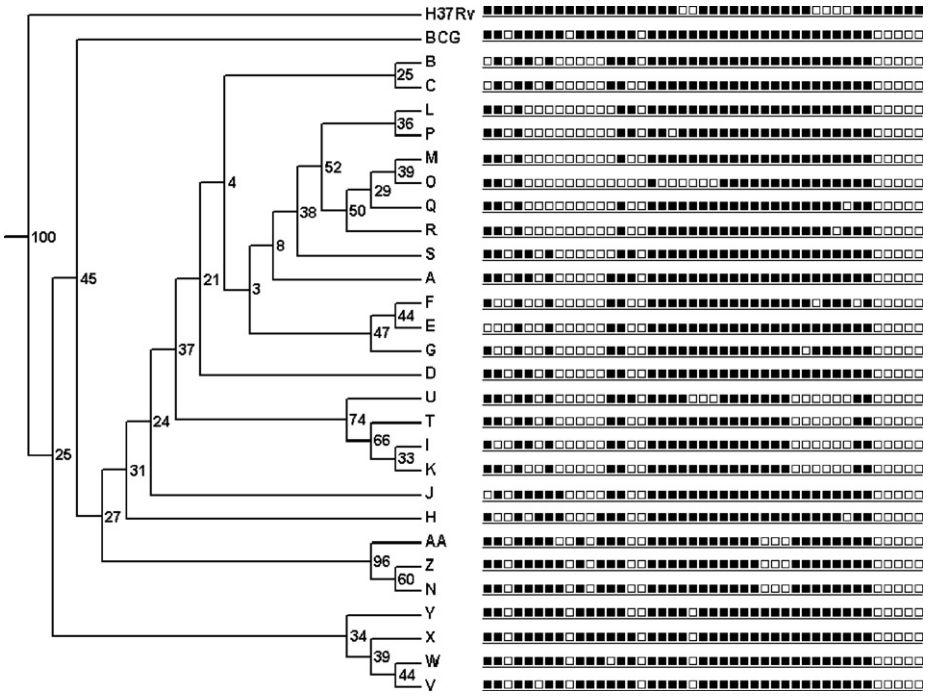

Fig. 1. Phylogenetic distribution by clusters (A to AA) of 84 spoligotypes of *M. bovis* strains obtained from cattle and wildlife in Mexico and the United States. Phylogenetic tree is rooted using *M. tuberculosis* strain H37Rv. *M. bovis* BCG is included as a reference strain. Bootstrap support at each node is represented as a percentage of the 1000 trees in which the group consisting of the species to the right occurred.

39–43, which allows differentiating *M. bovis* from *M. tuberculosis*, and the lack of spacers 3, 9 and 16. The distribution of spoligotypes in Fig. 1 is according to the dendrogram resulting from the cluster analysis performed by PHYLIP.

Spoligotypes resulting from the molecular analysis were grouped into 27 clusters, by convenience named from A to AA, ranging from 1 to 18 strains (Table 2). Cluster A was formed by 5 strains, two from Mexico and three from the US. All of the strains came from beef cattle in Texas, or were of unknown origin. Two of the animals of unknown origin were traced back to aguscalientes, Mexico, which indicates that they most probably were beef cattle, since dairy cattle are not exported to the US. Cluster B involved 8 strains, with all but one originating from TX and CA in the US, and were primarily from dairy cattle in Texas, or unknown origin. The Mexican isolate was also of unknown origin, recovered from a cow in BC.

Nine strains formed Cluster C; eight were from different states in Mexico (CHI, NL, JAL, TAM and COA) and one from CA in the US. Four of the strains in this cluster were beef cattle, three were dairy cattle, and two were of unknown origin. Cluster D was formed by 6 strains from the states of NL, SON, JAL and CHI in Mexico. Again, no distinct relationships regarding beef and dairy cattle could be observed, as two of the strains were recovered from cattle of unknown breed, two came from beef and two were from dairy cattle from different regions of Mexico.

Clusters E through K represented single isolates from AGS, CHI, JAL, NL and TAM in Mexico. E, F and G all grouped together within the phylogenetic tree, and represent spoligotypes that differ only in the presence or absence of single spacers at Locus 1, 32, 33 or 37. The spoligotypes represented by I and K are very similar as well, with the presence of a single spacer region at Locus 2 and 5 separating these strains. Spoligotype patterns of H and J are slightly more divergent, and differ at 5 spacer regions: Locus 1, 2, 5, 12 and 36.

Cluster L is the largest cluster in this analysis, containing 18 isolates. Fourteen of these strains came from the state of Michigan in the US, eight from cattle (6 beef and 2 dairy), five from deer and one from a Coyote. Genetic similarity of strains from different species in this cluster suggests transmission of TB between cattle and wildlife in Michigan. Strains from three feral pigs from Hawaii fell into this group, although no epidemiological information was available to explain this genetic association. Similarly, no epidemiological evidence is available to link the single NM isolate with the isolates from MI or HI.

Cluster M is the second largest cluster represented in the phylogenetic tree, and contains 12 different isolates, with 11/12 originating from five different states in Mexico (Table 2). The single US isolate in this cluster was recovered from a dairy cow in NM. The remaining clusters (N through AA) are again represented by single isolates with the exception of Cluster W, which contains four strains derived from a single outbreak in a dairy herd from CA (Table 2). Only 1 of these remaining 13 isolates is represented by a strain from the US; Cluster V contains a single isolate recovered from an elk in Montana which had epidemiological links to Canada (Robert Meyer, personal communication).

The topology of the phylogenetic tree supports the pairwise comparison analysis, in that isolates present within discrete clusters tend to be located from specific geographical regions. For example, the isolates from Cluster L were derived from animals located within the US that, with the exception of the single animal from NM, would have minimal, if any chance for contact with animals from Mexico based on geography alone. Genetic variability was more noticeable in *M. bovis* strains from Mexico than in those from the US; a total of 33 different spoligotypes were obtained with Mexican strains for only 9 from US strains. This might well be a consequence of a higher prevalence of the disease in Mexico.

Table 2
Epidemiology of strains from *M. bovis* isolates originating from the United States and Mexico, clustered by spoligotype designation

Spoligotype designation	Isolate	Country	State	Source	Species	Breed
A	02-0252	USA	TX	AGS, MX	Cattle	Unknown
	02-4192	USA	TX	AGS, MX	Cattle	Unknown
	02-5651	USA	AZ	Unknown	Cattle	Unknown
	03-4181	USA	TX	Herd # 5	Cattle	Beef
	03-5025	USA	TX	Herd # 5	Cattle	Beef
B	01-5950	USA	TX	Herd # 3	Cattle	Beef
	02-1652	USA	TX	Herd # 4	Cattle	Dairy
	02-4170	USA	CA	Herd # 2	Cattle	Dairy
	02-4544	USA	CA	Herd # 2	Cattle	Dairy
	02-6570	USA	CA	Unknown	Cattle	Dairy
	04-2067	USA	TX	Herd # 6	Cattle	Dairy
	04-6082	USA	CA	Unknown	Cattle	Dairy
	41	MX	BC	Unknown	Cattle	Unknown
C	13	MX	CHI	Rivapalacio	Cattle	Dairy
	02-2284	USA	CA	Herd # 2	Cattle	Dairy
	21	MX	NL	Chapotes	Cattle	Beef
	30	MX	JAL	Tzatlan	Cattle	Dairy
	35	MX	NL	Buysicobe	Cattle	Beef
	39	MX	NL	Adjuntas	Cattle	Beef
	46	MX	NL	Unknown	Cattle	Unknown
	48	MX	TAM	Tampio	Cattle	Beef
	44	MX	COA	Saltillo	Cattle	Unknown
D	50	MX	NL	Puntagua	Cattle	Beef
	6	MX	SON	Hilo	Cattle	Beef
	9	MX	JAL	Ameca	Cattle	Unknown
	10	MX	CHI	Jiminez	Cattle	Dairy
	31	MX	JAL	Tepatitlan	Cattle	Dairy
	42	MX	NL	Vmorelos	Cattle	Unknown
E	32	MX	AGS	Sfcorincon	Cattle	Dairy
F	8	MX	CHI	Unknown	Cattle	Unknown
G	12	MX	CHI	Jiminez	Cattle	Dairy
H	28	MX	JAL	Snmiguelito	Cattle	Dairy
I	47	MX	NL	Nutria	Cattle	Beef
J	40	MX	TAM	Nlaredo	Cattle	Unknown
K	24	MX	TAM	Guadalupe	Cattle	Beef
L	95-1315	USA	MI	Wildlife	Deer	N/A
	97-0214	USA	MI	Wildlife	Deer	N/A
	98-0415	USA	MI	Wildlife	Deer	N/A
	99-0440	USA	MI	Wildlife	Deer	N/A
	99-0745	USA	MI	Wildlife	Deer	N/A
	99-3877	USA	HI	Wildlife	Feral pig	N/A
	02-1372	USA	HI	Wildlife	Feral pig	N/A
	02-4263	USA	MI	Herd # 31	Cattle	Beef
	02-6234	USA	MI	Herd # 22	Cattle	Dairy
	02-6627	USA	MI	Herd # 24	Cattle	Dairy
	03-0751	USA	MI	Herd # 17	Cattle	Beef
	03-0752	USA	MI	Herd # 17	Cattle	Beef

Table 2 (*Continued*)

Spoligotype designation	Isolate	Country	State	Source	Species	Breed
	03-1057	USA	MI	Herd # 25	Cattle	Beef
	03-2069	USA	MI	Herd # 26	Cattle	Beef
	03-2070	USA	MI	Herd # 26	Cattle	Beef
	03-2859	USA	NM	Herd # 2	Cattle	Dairy
	03-5734	USA	HI	Wildlife	Feral pig	N/A
	05-2438	USA	MI	Wildlife	Coyote	N/A
M	03-5416	USA	NM	Herd # 2	Cattle	Dairy
	2	MX	SON	Sanluisb	Cattle	Beef
	3	MX	SON	Huatabampc	Cattle	Dairy
	4	MX	SON	Sanluisb	Cattle	Dairy
	5	MX	SON	Bacobampo	Cattle	Dairy
	17	MX	TAM	Ebanito	Cattle	Beef
	20	MX	TAM	Manantiales	Cattle	Beef
	26	MX	TAM	Ebanito	Cattle	Beef
	29	MX	AGS	Asientos	Cattle	Beef
	34	MX	SON	Buaysicobe	Cattle	Beef
	37	MX	BC	Tecate	Cattle	Beef
	38	MX	BC	Sanfelipe	Cattle	Dairy
N	49	MX	NL	Nutria	Cattle	Beef
O	14	MX	CHI	Rivapalacio	Cattle	Unknown
P	43	MX	BC	Sanfelipe	Cattle	Beef
Q	22	MX	COA	Torreon	Cattle	Dairy
R	45	MX	TAM	Manantiales	Cattle	Beef
S	05-1687	USA	TX	TAM, MX	Cattle	Unknown
T	15	MX	NL	Monterrey	Cattle	Beef
U	04-8012	USA	TX	COL, MX	Cattle	Unknown
V	91-2198	USA	MT	Wildlife	Elk	Unknown
W	03-1852	USA	CA	Herd # 1	Cattle	Dairy
	03-2616	USA	CA	Herd # 1	Cattle	Dairy
	03-2620	USA	CA	Herd # 1	Cattle	Dairy
	03-4006	USA	CA	Herd # 1	Cattle	Dairy
X	03-0248	USA	NM	Herd # 1	Cattle	Dairy
Y	36	MX	BC	Sanfelipe	Cattle	Dairy
Z	18	MX	BC	Canadagde	Cattle	Unknown
AA	19	MX	NL	Monterrey	Cattle	Unknown

4. Discussion

We have shown the usefulness of molecular techniques in resolving disputes between livestock-trade countries because of tuberculosis. Fingerprints of *M. bovis* strains indicate that in fact some animals from Mexico cross the Mexican–US border as false negatives, but also show that the US has its own source of infection, mostly in dairy cattle and wild-life.

The probability of contact between Mexican cattle and those populations from were *M. bovis* isolates were obtained in the US is low. On the one hand, fifteen of the 39 (38%) strains from the US analyzed in this study belong to dairy cattle, and 9 (23%) to wildlife. These are populations not expected to have contact with Mexican animals, which usually go to finishing feedlots in the

southern US states prior to being sent to slaughter. Therefore, the probability of Mexican animals being the source of infection for wildlife, dairy and beef cattle, especially in Michigan is not plausible. On the other hand, four (10%) of the beef cattle found with TB lesions in the US were traced back to Mexico, one of these strains (04-8012) shows a fingerprint identical to other spoligotypes found in Mexican isolates, suggesting Mexico as the place of infection. The other three strains from the US that trace back to Mexico (02-0252, 02-4192 and 05-1687) have unique spoligotype patterns, making difficult to identify the most probable source of infection.

A difference in possible sources of infection for cattle from both countries has been reported previously (Perumaalla et al., 1996). In this study using RFLP it was shown that strains from Mexico and the US had different fingerprints; although similarity between some strains from the two countries was observed, there were also strains from Texas with patterns similar to those observed in wildlife, suggesting transmission between wild-life species and cattle in that state.

Fingerprints similarities of *M. bovis* strains from both countries are evident in some clusters. These are likely the consequence of evolutionary divergence from the same ancestral progenitor, coupled with minimal restrictions for livestock trade dating back 50 years or more. The US has been selling dairy heifers for replacements to Mexico for many years without the trade requirement of a negative tuberculosis test, while Mexico has been exporting an average of 1.4 million beef calves annually to the US for fattening. Therefore, TB between Mexico and the US is an example of a disease that two commercial partner countries have to address jointly, as they have been doing since 1994, when the Mexican–American Bi-national Committee for the Eradication of Bovine Tuberculosis and Brucellosis was established.

M. bovis strains from Mexico show higher genetic diversity than those from the US. This might be a consequence of the unrestricted movement of animals between geographic locations within Mexico, and to the fact that TB is endemic in the country. Our results agree with previous studies in which high genetic diversity of Mexican isolates have been reported (Milian-Suazo et al., 2002, Santillan-Flores et al., 2006). Contrary to what occurs in other countries, such as Great Britain and Ireland, where geographical distribution of spoligotypes is observed, no such correlation between spoligotype patterns and geographic locations is seen in Mexico. As a consequence the current ''test and slaughter'' policies in Great Britain and Ireland based on restriction of animals' movement, this allows for a ''population sampling effect'' in these (Hewinson et al., 2006). In Mexico the ''test and slaughter'' strategy, although mandatory, can not always be enforced because of lack of compensation to producers. Therefore, heterogeneity of spoligotypes in Mexico most probably comes from exchange of strains between regions.

In the US the number of spoligotypes is much smaller than that in Mexico, correlating to the low prevalence of the disease in that country, in fact the US is considered a TB-free country. So, even though exchange of animals exists, the chance of spreading the disease is quite low. In relation to TB in Michigan, this situation is concerning for the US, and similarity between spoligotype patterns from cattle and wildlife indicate that infection between these animal species is occurring. In the long run TB in wildlife might be more difficult to eradicate than in cattle; transmission of strains from cattle to wildlife seems to increase the number of mutations because of adaptation of *M. bovis* to a new host and the change to a new genotype.

The genetic similarity observed between strains from feral pigs in Hawaii and cattle in Michigan, is not surprising; as with any genetic system, homeoplasy, or similarities between strains that are derived through independent mutation events with no epidemiological links, occurs at some level of frequency. When we compared our spoligotypes to those reported in www.Mbovis.org identical fingerprints were found, even though no relation exists between the

animal populations. This is why epidemiological information is so important to support molecular results in making valid conclusions about source of infection in outbreaks of TB.

5. Conclusions

Molecular epidemiology has shown to be a useful tool for preventing disputes between trade-partner countries. It has been shown that although some Mexican animals might cross the border infected, since they show a fingerprint characteristic of *M. bovis* strains in Mexico, the US has its own source of infection, mainly in dairy cattle and wildlife.

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References

Allix, C., Walravens, K., Saegerman, C., Godfroid, J., Supply, P., Fauville-Dufaux, M., 2006. Evaluation of the epidemiological relevance of variable-number tandem repeat genotyping of *Mycobacterium bovis* and comparison of the method with IS6110 restriction fragment length polymorphism analysis and spoligotyping. J. Clin. Microbiol. 44 (6), 1951–1962.

Aranaz, A., Liébana, E., Mateos, A., Domínguez, L., Vidal, D., Domingo, M., González, O., Rodríguez-Ferri, E.F., Bunschoten, A.E., van Embden, J.D.A., Cousins, D., 1996. Spacer oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: a tool for studying epidemiology of tuberculosis. J. Clin. Microbiol. 34, 2734–2740.

Collins, D.M., de Lisle, G.W., Gabric, D.M., 1986. Geographic distribution of restriction types of *Mycobacterium bovis* isolates from brush-tailed possums (Trichosurus vulpecula) in New Zealand. J. Hyg. (Camb.) 96, 431–438.

Collins, D.M., Gabric, D.M., de Lisle, G.W., 1988. Typing of *Mycobacterium bovis* isolates from cattle and other animals in the same locality. NZ Vet. J. 36, 45–46.

Cousins, D., Williams, S., Liébana, E., 1998. Evaluation of four DNA typing techniques in epidemiolgoical investigactions of bovine tuberculosis. J. Clin. Microbiol. 36 (1), 168–178.

de Lisle, G.W., Collins, D.M., Loveday, A.S., Young, W.A., Julian, A.F., 1990. Report of tuberculosis in cats in New Zealand and the examination of strains of *Mycobacterium bovis* by DNA restriction endonuclease analysis. NZ Vet. J. 38, 10–13.

Durr, P.A., Clifton-Hadley, R.S., Hewinson, R.G., 2000. Molecular epidemiology of bovine tuberculosis II. Applications of genotyping. Rev. Sci. Tech. Off. Int. Epiz. 19 (3), 689–701.

Farris, J.S., 1977. Phylogenetic analysis under Dollo's Law. Syst. Zool. 26, 77–88.

Felsenstein, J., 1989. PHYLIP—phylogenetic inference package (version 3.2). Cladistics 5, 164–166.

Frothingham, R., Meeker-O'connell, W.A., 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144, 1189–1196.

Grange, J.M., Yates, M.D., 1994. Zoonotic aspects of *Mycobacterium bovis* infection. Vet. Microbiol. 40, 137–151.

Hewinson, R.G., Vordermeier, H.M., Smith, N., Gordon, S.V., 2006. Recent advances in our knowledge of *Mycobacterium bovis*: a feeling for the organism. Vet. Microbiol. 112, 127–139.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. 35, 907–914.

Liébana, E., Aranaz, A., Domínguez, L., Mateos, A., González-Llamazarez, P., Rodríguez-Ferri, E.F., Domingo, M., Vidal, D., Cousins, D., 1995. Simple and rapid detection of *Mycobacterium* tuberculosis complex organism in bovine tissue samples by PCR. J. Clin. Microbiol. 33, 33–36.

Maslow, J.N., Mullingan, M.E., 1993. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clin. Infect. Dis. 17, 153–164.

Milian-Suazo, F., Banda-Ruiz, V., Ramirez-Casillas, C., Arriaga-Diaz, C., 2002. Genotyping of *Mycobacterium bovis* by geographic location within Mexico. Prev. Vet. Med. 55, 255–264.

Murray, C.J.L., Styblo, K., Rouillon, A., 1990. Tuberculosis in developing countries:burden, intervention and cost. Tuber. Lung Dis. 65, 2–20.

Perumaalla, V.S., Adams, L.G., Payeur, J.B., Jarnagin, J.L., Baca, D.R., Guemes, F.S., y Ficht, T.A., 1996. Molecular epidemiology of *Mycobacterium bovis* in Texas and Mexico. J. Clin. Microbiol. 34 (9), 2066–2071.

Perumaalla, V.S., Adams, L.G., Payeur, J., Baca, D., Ficht, T.A., 1999. Molecular fingerprinting confirms extensive cow-to-cow intra-herd transmission of a single *Mycobacterium bovis* strain. Vet. Microbiol. 70, 269–276.

Roring, S., Scott, A.N., Glyn Hewinson, R., Neill, S.D., Skuce, R.A., 2004. Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of *Mycobacterium bovis* isolates from Ireland. Vet. Microbiol. 101, 65–73.

Santillan-Flores, M.A., Flores, J., Arriaga-Diaz, C., Romero-Torres, C., Suárez-Güemes, F., Espitia, C., 2006. Polymorphism of the PE domain of PE/PE_PGRS sequences in clinical isolates of *Mycobacterium bovis* in Mexico. Vet. Microbiol. 115, 364–369.

Saunders, N.A., 1991. Análisis of restriction fragment length polimorphism in the study of bacteria. In: Grange, J.M., Fox, A., Morgan, N.L. (Eds.), Genetic Manipulation Techniques and Applications. Society for Applied Bacteriology Technical Series. No. 28. Blackwell, Oxford, pp. 227–244.

Szewzyk, R., Venson, S.B., Hoffner, S.E., Bolske, G., Wahstrom, H., Englund, L., Engvall, A., Kalenius, G., 1995. Molecular epidemiological studies of *Mycobacterium bovis* infections in humans and animals in Sweden. J. Clin. Microbiol. 33, 3183–3185.

van Soolingen, D., de Haas, P.E.W., Haagsma, J., Eger, T., Hermans, P.W.M., Ritacco, V., Alito, A., van Embden, J.D.A., 1994. Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. J. Clin. Microbiol. 32, 2425–2433.